HEXADECANE-INDUCED HYPERKERATINIZATION OF GUINEA PIG SKIN

I. Changes in Epidermal Weight and Water Binding*

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It has been reported (1–5) that topical application of various noncarcinogenic hydrocarbons and mineral oils to mammalian skin leads to hyperplasia and hyperkeratinization. Of the saturated, straight-chained hydrocarbons (alkanes), hexadecane is reported to induce the most marked changes in the skin of guinea pigs (3, 5). In rough chronological order, the changes which occur following hexadecane treatment are (3): reddening (which is not reflected in any histologically-apparent inflammatory state), cellular hypertrophy, marked increases in epidermal mitotic activity, hyperplasia of the vital layers, marked hyperkeratinization of the epidermal surface and hair follicles with an abnormal type of keratin, and, eventually, desquamation of large keratinized flakes with return of the skin to a nearly normal—if somewhat thinned and epilated—state.

This paper is the first in a series of reports of certain quantitative changes in the properties and composition of guinea pig epidermis during the reaction to hexadecane. Such work was undertaken because it was felt that direct observation of temporal changes in epidermis undergoing marked changes in the level and type of keratinization in response to a known stimulus should eventually provide more meaningful information about the mechanism of keratinization than deductions from observation of spatially-separated events (*e.g.* histological studies of normal epidermis or comparative chemical studies of keratins of various origins).

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METHODS

Experimental animals. All of the work reported herein was carried out using male albino guinea pigs weighing 700 to 800 gm, housed individually in hanging, screen-bottom cages in an air-conditioned, humidity-controlled room and maintained on commercial vitamin C fortified guinea pig pellets and distilled water *ad libitum*. Daily oral supplements of ascorbic acid were administered during the experimental period, when the vitamin C requirement might be expected to increase.

Except where a specific exception is made below, all animals were treated with hexadecane in a similar manner. On day one of an experimental period all guinea pigs were closely clipped on both sides with a small animal clipper, leaving a strip of hair on dorsal and ventral surfaces. The left side of each animal was then treated with purified n-hexadecane, obtained from American Oil Company, Research and Development Department, Whiting, Indiana. Originally treatments were at the level of two ml per kilogram body weight, sprayed on with an atomizer. Subsequently it was found that the same effect could be obtained with greater reproducibility by applying 1.5 ml per kilogram in a fine stream from a 25 gauge needle fitted to a hypodermic syringe; thus all but the earliest treatments were so performed. Treatments were repeated on days three and five. The right side of the animal was in all cases left untreated to serve as an individual control. Hair was reclipped prior to killing the animal.

As will be noted below, in one series of experiments treatments were made only on day one, to circumvent difficulties in chronology inherent in the usual treatment schedule. Details of such treatments will be given in the text in the appropriate place.

All animals were killed by etherization. Immediately upon respiratory and cardiac failure animals were removed from the anesthesia chamber and the skin from both treated and control sides was excised free of subcutaneous tissue and chilled. All further manipulations were carried out between 0° and 5° C. unless otherwise noted.

Preparation of epidermal samples. Three different types of epidermal samples from control and treated skin were taken; samples of whole epidermis from known areas of skin (used for determining fresh and dry weight of epidermis per unit area and water binding of whole epidermis); samples of whole epidermis collected from the remaining excised areas (used for extraction studies etc.);

and epidermal scales or skin flakes (used to determine which of the changes observed in whole epidermis could be localized in the cornified layers).

All separations of dermis from epidermis were effected by the stretch method of Van Scott (6) which is clearly the method of choice when chemical integrity of the epidermis is a prime consideration. Since the method involves roughly a 1.5- to 2.5-fold displacement of mass per unit area during the separation, however, the difficulty of obtaining meaningful measurements of mass of epidermis per unit body surface area (a key value) is great. The device drawn in Figure 1 was eventually designed to carry out such a separation and was found to give precision in the range of ±8% for replicate one cm^2 epidermal samples taken at random from a single piece of skin. Precision was improved to ±5% if, after collecting epidermis of the stretched area, the exposed dermis and an adjacent area of whole skin were excised and the values for direct epidermal weight and that obtained by difference were averaged. Samples so obtained were placed in tared weighing bottles, weighed, frozen in dry ice, lyophilized, dried over night at 100° C. *in vacuo* over P_2O_5 and reweighed; thus yielding values for fresh and dry weight.

The portion of excised skin remaining after removal of such samples for dry weight determination was then tacked down at maximum distention over a chilled convex wooden surface and the remaining epidermis removed as quantitatively as possible by blunt dissection. This epidermal tissue was weighed and either extracted and/or analyzed at once or lyophilized pending subsequent use.

Quantitative separation of guinea pig epidermal scales (or stratum corneum) from a known area of epidermis has not been achieved in this laboratory; large quantities of such scales from indeterminate areas of treated and control skin were, however, relatively easy to obtain for comparative studies. Normal scales were obtained by pouring normal guinea pig fur clippings which had been slurred in diethyl ether onto a stacked series of 60 to 400 mesh sieves (*cf.* 7). Hairs accumulated at various levels of the stack, while epidermal scales passed the finest sieve and were collected on a lintless filter paper. Samples found on microscopic examination to contain appreciable quantities of short hair were discarded. Hyperkeratotic scales were simply plucked from the skin as they began to desquamate at about day 14 after the first hexadecane treatment; these scales were as much as thirty millimeters in diameter and a millimeter or more thick. Hyperkeratotic scales were exhaustively extracted with ether for purposes of subsequent comparison of their properties with those of ether-collected normal scales.

Water-binding studies. Samples of epidermis, scales, or dried extracts were placed in tared, glass-stoppered weighing bottles. The empty tare was always treated in a manner identical to the sample bottles. Samples, bottles and caps were

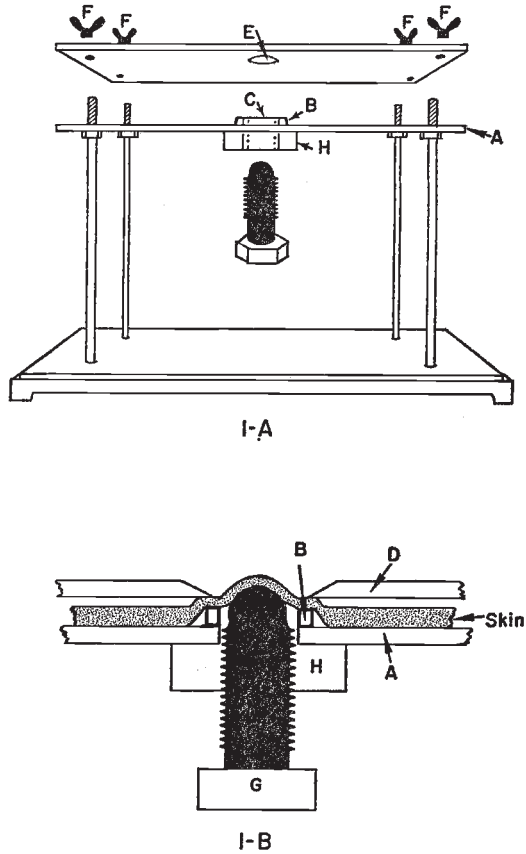

FIG. 1. *Device used for separation of epidermis from dermis in a known area.* 1a.: Perspective view of dissembled apparatus. 1b.: Cross section of working area with a sample of skin in place.

In use, excised skin is laid out at normal distention over lower plate (A), to which ring (B) is soldered around hole (C). Upper plate (D) with matching hole (E) is lowered into place above skin. When wing nuts (F) are tightened, skin is pinched firmly between ring (B) and upper plate (D). Then the brass bolt (G) with domed end is screwed up through nut (H) which is brazed to lower plate (A). This distends the skin which is exposed between the plates and ruptures the bond between dermis and epidermis, permitting separation (*cf.* 6). Hole (E) in upper plate is bevelled coliquely to permit easy dissection of epidermis to the very edges of the exposed area. Dimensions are arbitrary. Area of skin separated is determined by diameter of holes (C and E) which should be selected with sufficient clearance to prevent shearing of skin between (G) and (E). Authors used ½ inch bolt and an effective area of 1.25 cm^2.

first allowed to equilibrate to constant weight in a two liter desiccator over 500 ml of concentrated sulfuric acid (S.G. 1.840) at 37° C. and then were transferred to a second desiccator containing 500 ml 24% sulfuric acid (S.G. 1.160) and equilibrated once again to constant weight. Specific gravities of the acids were checked every time the desiccators

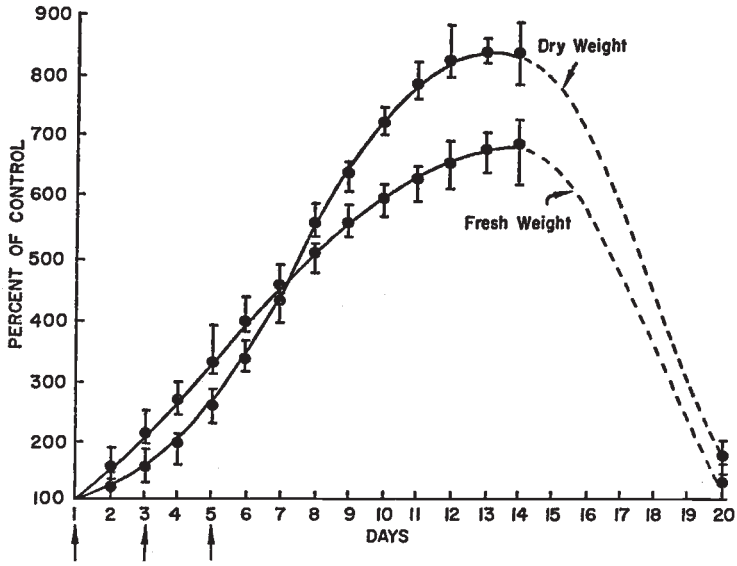

FIG. 2. *Change in epidermal weight of guinea pig skin with time after treatment with n-hexadecane.* Data plotted as per cent of contralateral control, *i.e.*, mg epidermis per cm^2 on treated side divided by mg epidermis per cm^2 on control side, multiplied by 100. Treatments at vertical arrows. Twenty-five animals used. Bars represent range.

TABLE I

Effect of n-hexadecane on weight (mg per cm^2) of guinea pig skin

	Control Side		Treated Side	
	Fresh Wgt	Dry Wgt	Fresh Wgt	Dry Wgt
Whole skin	163 ± 25	55 ± 10	295 ± 35	135 ± 15
Epidermis	24 ± 10	11 ± 4	148 ± 25	85 ± 11
Dermis	140 ± 15	45 ± 5	143 ± 15	49 ± 5

Figures are mean ± standard deviation for 37 animals treated on days 1, 3 and 5 and killed on day 12.

were opened and the acid was replaced as necessary. These concentrations of acid are in equilibrium, at 37° C, with air at 0% and 85% relative humidity, respectively (8). Thus a value for water-binding potential at 37° C. and 85% relative humidity could be readily obtained.

Fractionation of epidermis. Water extracts of epidermal tissue or scales from normal and hyperkeratotic skin were made by maceration of the pulverized, lyophilized tissue in 25 volumes of cold toluene-saturated distilled water with gentle agitation overnight. The resulting suspensions were centrifuged at 15,000 × *g* for 30 minutes. The residues were twice re-extracted and combined supernatants and wash of the final residue were clarified by filtration through fine sintered glass, and the filtrate taken to a convenient known volume in a volumetric flask. Aliquots were then taken for solids determination (by lyophilizing in a weighed bottle), dialysis and determination of water-binding potential of dialyzable and non-dialyzable fractions. Further fractionation of residues by extraction with protein solvents was then performed. Details of those procedures will be discussed in a subsequent paper (9).

RESULTS

Gross changes in skin appearance. The same series of changes were observed as were reported previously (3), namely: marked reddening shortly after the first treatment (which persisted until hyperkeratotic thickening of the skin obscured it), thickening and stiffening of the skin beginning about the third day and progressing to the fourteenth or fifteenth day, whereupon desquamation of large, thick scales commenced. All effects were strictly unilateral. Occasionally epidermal stiffening was sufficiently severe to lead to immobilization and thus debilitation.

Changes in epidermal weight. Figure 2 is a graphic representation of changes in fresh and dry epidermal weight with time after treatment in a typical experiment. The maximal effect (eight-fold increase in dry weight per unit area) occurred at about day 12. Originally the fresh epidermal weight increased more rapidly than the dry weight (due, probably to proliferation of the vital cell layers), but by day 7 the curves had crossed. As can be seen from Table I this resulted in a drop in water content of the epidermis from 65% to 40% in this twelve-day period. It is not clear from these data whether this decrease in apparent water content is due solely to a shift in ratio of cornified to vital cells, or a decreased water binding by the cornified cells. This problem will be considered below.

It is also apparent from Table I that although flushing of the skin was the first apparent response to hexadecane, there was no significant effect upon dermal weight per unit area. This was true for the entire duration of the experiment and is consistent with the observation that histological defects were not apparent in the dermis (3). Changes in chemical composition or physiology of the dermis are of course not hereby excluded.

In Table II data are summarized concerning the relationship of different hexadecane-treatment schedules on the degree of thickening of the epidermis. Several conclusions can be drawn from these data. First, guinea pigs responded to a single topical dose of n-hexadecane (groups III and IV) and the degree of reaction to a single dose was dose dependent (III vs. IV). Secondly, the effects of repeated doses were cumulative but much more than additive if the interval between doses was adequate. Thus group II showed a 550% increase in epidermal weight in response to three treatments, the first of which would lead to only a 75% increase! And, lastly, response was not directly proportional to total dose when the pattern of application varied, since a total dose of 6 ml applied in ten aliquots over a five hour period gave a much milder reaction than three-fourths that dose applied over a five day period. (Indeed, for this particular test animal—a 700 to 800 gm guinea pig—the bi-daily application of 1.5 ml gave about a maximal response.)

TABLE II

Effect of n-hexadecane-treatment pattern on guinea pig epidermal weight

Group Number	Number of Animals	Dose in ml/kg Body Weight	Number of Doses	Interdose Interval	Day of Sacrifice	Epidermal Dry Wt as % of Control
I	37	1.5	3	48 hrs.	12	800
II	6	1.5	3	48 hrs.	9	650
III	3	1.5	1	none	9	175
IV	3	2.0	1	none	9	210
V	6	0.6	10	30 min.	9	290

TABLE III

Water binding by epidermal scales and their derivatives*

All values in mg per 100 mg of dry scales

Fraction	Normal (Untreated)		Hyperkeratotic (Hexadecane-Treated)	
	Weight of fraction	Water bound	Weight of fraction	Water bound
Whole scales.....	100	47	100	30
Water insoluble.....	72	6	74	8
Water soluble.....	28	40	26	22
dialyzable.....	18	36	16	16
non-dialyzable....	9	3	10	3

* At 37° C. and 85% relative humidity.

Changes in water binding of epidermal scales. The question was posed above whether the decrease in apparent water content of the epidermis was due simply to a shift in the ratio of vital, relatively wet, to cornified, relatively dry cells or to a decreased water binding potential of the cornified cells themselves. This question cannot yet be answered quantitatively, since quantitative separation of epidermis into living and cornified layers over a known area has not yet been achieved. Nonetheless, as Table III and Figure 3 show, scales from hexadecane treated guinea pig skin had a much lower water binding potential than did scales from normal guinea pig skin. Thus it can be said that the hyperkeratinized epidermis was dryer because it was composed of a thicker layer of cornified material and because this cornified material had a lower water-binding potential.

Furthermore, it is apparent from both Table III and Figure 3 that the decreased water-binding potential of the hyperkeratotic scales was attributed to decreased water-binding by the water soluble, dialyzable fraction thereof. This fraction was decreased both in amount and in water binding potential per gram. Analysis of

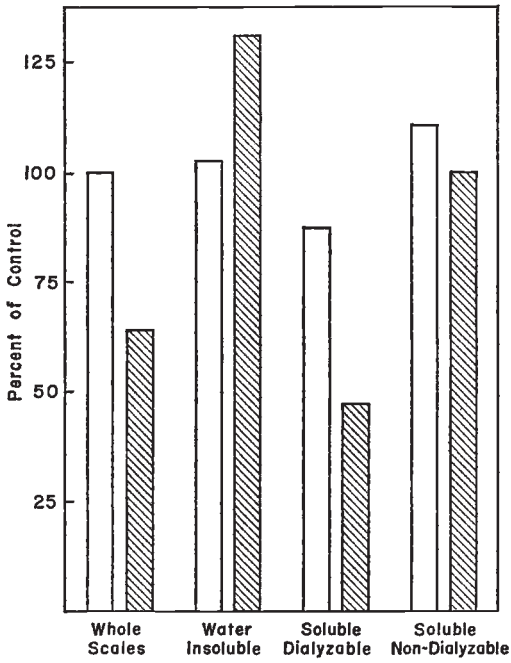

FIG. 3. *Dry weight and water binding by hyperkeratotic scales and their derivatives compared to control scales.* Measurements of water binding made at 85% relative humidity and 37° C. Data expressed as per cent of control, *i.e.*, mg per 100 mg hyperkeratotic scales divided by mg per 100 mg of normal scales, multiplied by 100. Open bars: weight of fraction; hatched bars: water bound by that fraction.

this fraction will be reported in a subsequent publication (10).

DISCUSSION

It has been shown herein that the hyperkeratinization which results from topical hexadecane treatment is reproducible and predictable, and provides a model for studying the keratinization process, since a rapid, dramatic, localized increase in the production of keratinized tissue can be induced by manipulation of a single variable. There are two primary reservations which must, however, be stated in recommending the system for investigation. The first is that hair regrowth in clipped areas on treated and control sides is markedly different, since on the treated side it is repressed by hyperkeratinization of the follicular orifices. Thus, if one is not alert, measurements might inadvertently be made on the differences between hair and surface keratinization. (In these experiments hair was reclipped prior to sacrifice of the animal.) Secondly, the matter of treatment chronology is unfortunate. Since maximal effects occur only if treatments are made on days one, three and five, studies of chronology of changes are much complicated. We have tried to circumvent this by studying a given aspect of the system in the three-treatment syndrome where changes are maximal and thereafter determining whether and when parallel changes occur in the milder one-treatment syndrome.

The decreased water-binding of the hyperkeratinized stratum corneum which coincides with decreased levels of and water-binding by the water-soluble, dialyzable fraction is of interest and lends added support to Blank's (11, 12) theory on skin water balance.

SUMMARY

This report concerned the hyperkeratotic response of guinea pig skin to topical n-hexadecane treatment as measured by epidermal weight per unit area. A device for separating epidermis from dermis in a known area of skin was described. There was a dose-dependent response to a single dose of n-hexadecane; repeated, properly spaced doses gave a greater than additive response; and the degree of response to multiple doses was more dependent on inter-dose interval than total dose. In all cases the effects were unilateral and in no case was there evidence of gross changes in the dermis other than reddening. Epidermal dry weight was increased by as much as 800% by application of three doses of n-hexadecane at 48 hour intervals.

Concurrent with the increase in epidermal weight there was a loss in water content of the epidermis. This decreased water content was due, at least in part, to a decreased water binding potential of the water-soluble, dialyzable fraction of the cornified layer.

Since the response of the epidermis is localized, dramatic and predictable, it is suggested that this system provides a model for study of the keratinization process.

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